METHODS OF EX-VIVO EXPANSION OF HEMATOPOEITIC CELLS USING INTERLEUKIN-3 (IL-3) MULTIPLE MUTATION POLYPEPTIDES

This is a continuation-in-part of United States Application Serial No. 08/411,795 filed November 22, 1993 which a continuation-in-part of United States Application Serial No. 07/981,044 filed November 24, 1992, which is now abandonded, which are incorporated herein by reference.

Field of the Invention

The present invention relates to methods of ex-vivo expansion of hematopoietic cells by culturing hematopoietic cells in a growth medium comprising a variant of human interleukin-3 (hIL-3) which contains multiple amino acid substitutions and which may have portions of the native hIL-3 molecule deleted. These hIL-3 multiple mutation polypeptides retain one or more activities of native hIL-3 and may also show improved hematopoietic cell-stimulating activity and/or an improved activity profile which may include reduction of undesirable biological activities associated with native hIL-3. The present invention also relates to the use of the expanded hematopoietic cells for treating patients having a hematopoietic disorder.

Background of the Invention

Colony stimulating factors (CSFs) which stimulate the differentiation and/or proliferation of bone marrow cells have generated much interest because of their therapeutic potential for restoring depressed levels of hematopoietic stem cell-derived cells. CSFs in both human and murine systems have been identified and

distinguished according to their activities. For example, granulocyte-CSF (G-CSF) and macrophage-CSF (M-CSF) stimulate the in vitro formation of neutrophilic granulocyte and macrophage colonies, respectively while GM-CSF and interleukin-3 (IL-3) have broader activities and stimulate the formation of both macrophage, neutrophilic and eosinophilic granulocyte colonies. IL-3 also stimulates the formation of mast, megakaryocyte and pure and mixed erythroid colonies.

Because of its ability to stimulate the proliferation of a number of different cell types and to support the growth and proliferation of progenitor cells, IL-3 has potential for therapeutic use in restoring hematopoietic cells to normal amounts in those cases where the number of cells has been reduced due to diseases or to therapeutic treatments such as radiation and chemotherapy.

Interleukin-3 (IL-3) is a hematopoietic growth factor which has the property of being able to promote the survival, growth and differentiation of hematopoietic cells. Among the biological properties of IL-3 are the ability (a) to support the growth and differentiation of progenitor cells committed to all, or virtually all, blood cell lineages; (b) to interact with early multipotential stem cells; (c) to sustain the growth of pluripotent precursor cells; (d) to stimulate proliferation of chronic myelogenous leukemia (CML) cells; (e) to stimulate proliferation of mast cells, eosinophils and basophils; (f) to stimulate DNA synthesis by human acute myelogenous leukemia (AML) cells; (g) to prime cells for production of leukotrienes and histamines; (h) to induce leukocyte chemotaxis; and (i) to induce cell surface molecules needed for leukocyte adhesion.

Mature human interleukin-3 (hIL-3) consists of 133 amino acids. It has one disulfide bridge and two potential glycosylation sites (Yang, et al., *CELL* **47**:3,

1986).

Murine IL-3 (mIL-3) was first identified by Ihle, et al., (*J. IMMUNOL.* **126**:2184, 1981) as a factor which induced expression of a T cell associated enzyme, 20`-hydroxysteroid dehydrogenase. The factor was purified to homogeneity and shown to regulate the growth and differentiation of numerous subclasses of early hematopoietic and lymphoid progenitor cells.

In 1984, cDNA clones coding for murine IL-3 were isolated (Fung, et al., *NATURE* **307**:233, 1984; Yokota, et al., *PROC. NATL. ACAD. SCI. USA* **81**:1070, 1984). The murine DNA sequence coded for a polypeptide of 166 amino acids including a putative signal peptide.

The gibbon IL-3 sequence was obtained using a gibbon cDNA expression library. The gibbon IL-3 sequence was then used as a probe against a human genomic library to obtain a human IL-3 sequence.

Gibbon and human genomic DNA homologues of the murine IL-3 sequence were disclosed by Yang, et al., (*CELL* **47**:3, 1986). The human sequence reported by Yang, et al., included a serine residue at position 8 of the mature protein sequence. Following this finding, others reported isolation of Pro^8 hIL-3 cDNAs having proline at position 8 of the protein sequence. Thus it appears that there may be two allelic forms of hIL-3.

Dorssers, et al., (*GENE* **55**:115, 1987), found a clone from a human cDNA library which hybridized with mIL-3. This hybridization was the result of the high degree of homology between the 3' noncoding regions of mIL-3 and hIL-3. This cDNA coded for an hIL-3 (Pro^8) sequence.

U.S. 4,877,729 and U.S. 4,959,454 disclose human IL-3 and gibbon IL-3 cDNAs and the protein sequences for which they code. The hIL-3 disclosed has serine rather than proline at position 8 in the protein sequence.

Clark-Lewis, et al., (*SCIENCE* **231**:134, 1986) performed a functional analysis of murine IL-3 analogues

synthesized with an automated peptide synthesizer. The authors concluded that the stable tertiary structure of the complete molecule was required for full activity. A study on the role of the disulfide bridges showed that replacement of all four cysteines by alanine gave a molecule with 1/500th the activity as the native molecule. Replacement of two of the four Cys residues by Ala(Cys^{79}, Cys^{140} -> Ala^{79}, Ala^{140}) resulted in an increased activity. The authors concluded that in murine IL-3 a single disulfide bridge is required between cysteines 17 and 80 to get biological activity that approximates physiological levels and that this structure probably stabilizes the tertiary structure of the protein to give a conformation that is optimal for function. (Clark-Lewis, et al., *PROC. NATL. ACAD. SCI. USA* **85**:7897, 1988).

International Patent Application (PCT) WO 88/00598 discloses gibbon- and human-like IL-3. The hIL-3 contains a Ser^{8} -> Pro^{8} replacement. Suggestions are made to replace Cys by Ser, thereby breaking the disulfide bridge, and to replace one or more amino acids at the glycosylation sites.

EP-A-0275598 (WO 88/04691) illustrates that Ala^{1} can be deleted while retaining biological activity. Some mutant hIL-3 sequences are provided, e.g., two double mutants, Ala^{1} -> Asp^{1}, Trp^{13} -> Arg^{13} (pGB/IL-302) and Ala^{1} -> Asp^{1}, Met^{3} -> Thr^{3} (pGB/IL-304) and one triple mutant Ala^{1} -> Asp^{1}, Leu^{9} -> Pro^{9}, Trp^{13} -> Arg^{13} (pGB/IL-303).

WO 88/05469 describes how deglycosylation mutants can be obtained and suggests mutants of Arg^{54}Arg^{55} and Arg^{108}Arg^{109}Lys^{110} might avoid proteolysis upon expression in Saccharomyces cerevisiae by KEX2 protease. No mutated proteins are disclosed. Glycosylation and the KEX2 protease activity are only important, in this context, upon expression in yeast.

WO 88/06161 mentions various mutants which theoretically may be conformationally and antigenically

neutral. The only actually performed mutations are Met^{2} -> Ile^{2} and Ile^{131} -> Leu^{131}. It is not disclosed whether the contemplated neutralities were obtained for these two mutations.

WO 91/00350 discloses nonglycosylated hIL-3 analog proteins, for example, hIL-3 ($Pro^{8}Asp^{15}Asp^{70}$), Met^{3} rhul-3 ($Pro^{8}Asp^{15}Asp^{70}$); Thr^{4} rhuL-3 ($Pro^{8}Asp^{15}Asp^{70}$)and Thr^{6} rhuIL-3 ($Pro^{8}Asp^{15}Asp^{70}$). It is said that these protein compositions do not exhibit certain adverse side effects associated with native hIL-3 such as urticaria resulting from infiltration of mast cells and lymphocytes into the dermis. The disclosed analog hIL-3 proteins may have N termini at Met^{3}, Thr^{4}, or Thr^{6}.

WO 91/12874 discloses cysteine added variants (CAVs) of IL-3 which have at least one Cys residue substituted for a naturally occurring amino acid residue.

Hematopoietic growth factors, such as hIL-3, have been administered alone, co-administered with other hematopoietic growth factors, or in combination with bone marrow transplants subsequent to high dose chemotherapy to treat the neutropenia and thrombocytopenia which are often the result of such treatment. However the period of severe neutropenia and thrombocytopenia may not be totally eliminated. The myeloid lineage, which is comprised of monocytes (macrophages), granulocytes (including neutrophils) and megakaryocytes, is critical in preventing infections and bleeding which can be life-threatening. Neutropenia and thrombocytopenia may also be the result of disease, genetic disorders, drugs, toxins, radiation and many therapeutic treatments such as conventional oncology therapy.

Bone marrow transplants have been used to treat this patient population. However, several problems are associated with the use of bone marrow to reconstitute a compromised hematopoietic system including: 1) the

number of stem cells in bone marrow or other is limited, 2) Graft Versus Host Disease, 3) graft rejection and 4) possible contamination with tumor cells. Stem cells make up a very small percentage of the nucleated cells in the bone marrow, spleen and peripheral blood. It is clear that a dose response exits such that a greater number of stem cells will enhance hematopoietic recovery. Therefore, the use of stem cells, that have been expanded ex-vivo, should enhance hematopoietic recovery and patient survival. Bone marrow from an allogeneic donor has been used to provide bone marrow for transplant. However, Graft Versus Host Disease and graft rejection limit bone marrow transplantation even in recipients with HLA-matched sibling donors. An alternative to allogenic bone marrow transplants is autologous bone marrow transplants. In autologous bone marrow transplants, some of the patient's own marrow is harvested prior to myeloablative therapy, e.g. high dose chemotherapy, and is transplanted back into the patient afterwards. Autologous transplants eliminate the risk of Graft Versus Host Disease and graft rejection. However, autologous bone marrow transplants still present problems in terms of the limited number of stems cells in the marrow and possible contamination with tumor cells.

The limited number of stem cells may be overcome by ex-vivo expansion of the stem cells. In addition, stem cells can be specifically isolated selected based on the presence of specific surface antigen such as CD34+ in order to decrease tumor cell contamination of the marrow graft.

The following patents contain further details on separating stem cells, CD34+ cells, culturing the cells with hematopoietic factors, the use of the cells for the treatment of patients with hematopoietic disorders and the use of hematopoietic factors for cell expansion and

gene therapy.

5,061,620 relates to compositions comprising human hematopoietic stem cells provided by separating the stem cells from dedicated cells.

5,199,942 describes a method for autologous hematopoietic cell transplantation comprising: (1) obtaining hematopoietic progenitor cells from a patient; (2) ex-vivo expansion of cells with a growth factor selected from the group consisting of IL-3, flt3 ligand, c-kit ligand, GM-CSF, IL-1, GM-CSF/IL-3 fusion protein and combinations thereof; (3) administering cellular preparation to a patient.

5,240,856 relates to a cell separator that includes apparatus for automatically controlling the cell separation process.

5,409,813 describes methods of positive and negative selection of a cell population from a mixture of cell populations utilizing a magnetically stabilized fluidized bed.

5,409,825 relates to a method of growing hematopoietic stem cells in a liquid culture medium using mast cell growth factor (MGF) and optionally at least one cytokine selected from the group consisting of IL-3, GM-CSF and IL-3/GM-CSF fusion protein.

5,459,069 relates to devices for maintaining and growing human stem cells and/or hematopoietic cells in culture.

5,541,103 describes peripheral blood progenitor cells obtained by enriching blood progenitors expressing the cd34 antigen and culture the cells in a growth medium consisting of IL-1, IL-3, IL-6, erythropoietin and stem cell growth factor.

5,464,753 describes a method of purifying pluripotent hematopoietic stem cells expressing P-glycoprotein from a mixture of blood or bone marrow cells.

5,547,687 relates to a method of enriching CD34 cells from a cell mixture by density centrifugation.

5,571,686 depicts the use of megapoietin (c-mpl ligand) for the in vitro expansion of stem cells as a source of platelets for transplantation and for increasing the storage life of platelets.

WO 91/16116 describes devices and methods for selectively isolating and separating target cells from a mixture of cells.

WO 91/18972 describes methods for in vitro culturing of bone marrow, by incubating suspension of bone marrow cells, using a hollow fiber bioreactor.

WO 92/18615 relates to a process for maintaining and expanding bone marrow cells, in a culture medium containing specific mixtures of cytokines, for use in transplants.

WO 93/08268 describes a method for selectively expanding stem cells, comprising the steps of (a) separating CD34+ stem cells from a mixed population of cells and (b) incubating the separated cells in a selective medium, such that the stem cells are selectively expanded.

WO 93/18136 describes a process for in vitro support of mammalian cells derived from peripheral blood.

WO 93/18648 relates to a composition comprising human neutrophil precursor cells with a high content of myeloblasts and promyelocytes for treating genetic or

acquired neutropenia.

WO 94/08039 describes a method of enrichment for human hematopoietic stem cells by selection for cells which express c-kit protein.

WO 94/11493 describes a stem cell population that are CD34+ and small in size, which are isolated using a counterflow elutriation method.

WO 94/27698 relates to a method combining immunoaffinity separation and continuous flow centrifugal separation for the selective separation of a nucleated heterogeneous cell population from a heterogeneous cell mixture.

WO 94/25848 describes a cell separation apparatus for collection and manipulation of target cells.

The long term culturing of highly enriched CD34+ precursors of hematopoietic progenitor cells from human bone marrow in cultures containing IL-1α, IL-3, IL-6 or GM-CSF is discussed in Brandt et al., *J. Clin. Invest.* 86:932-941, 1990.

Summary of the Invention

The present invention relates methods of ex-vivo expansion of hematopoietic cells by culturing hematopoietic cells in a growth medium comprising a recombinant human interleukin-3 (hIL-3) variant or mutant proteins (muteins). These hIL-3 muteins contain amino acid substitutions and may also have amino acid deletions at either/or both the N- and C- termini. Preferably, these mutant polypeptides of the present invention contain four or more amino acids which differ from the amino acids found at the corresponding

positions in the native hIL-3 polypeptide. The present invention includes mutants of hIL-3 in which from 1 to 14 amino acids have been deleted from the N-terminus and/or from 1 to 15 amino acids have been deleted from the C-terminus, and in which multiple amino acid substitutions have been made. Preferred muteins of the present invention are those in which amino acids 1 to 14 have been deleted from the N-terminus, amino acids 126 to 133 have been deleted from the C-terminus, and which also contain from about four to about twenty-six amino acid substitutions in the polypeptide sequence.

The present invention is directed to methods for ex vivo expansion of stem cells, comprising the steps of; (a) culturing said stem cells with a selected growth medium comprising a human interleukin-3 mutant polypeptide of (SEQ ID NO:15);

wherein Xaa at position 17 is Ser, Lys, Gly, Asp, Met, Gln, or Arg;
Xaa at position 18 is Asn, His, Leu, Ile, Phe, Arg, or Gln;
Xaa at position 19 is Met, Phe, Ile, Arg, Gly, Ala, or Cys;
Xaa at position 20 is Ile, Cys, Gln, Glu, Arg, Pro, or Ala;
Xaa at position 21 is Asp, Phe, Lys, Arg, Ala, Gly, Glu, Gln, Asn, Thr, Ser or Val;
Xaa at position 22 is Glu, Trp, Pro, Ser, Ala, His, Asp, Asn, Gln, Leu, Val or Gly;
Xaa at position 23 is Ile, Val, Ala, Leu, Gly, Trp, Lys, Phe, Ser, or Arg;
Xaa at position 24 is Ile, Gly, Val, Arg, Ser, Phe, or Leu;
Xaa at position 25 is Thr, His, Gly, Gln, Arg, Pro, or Ala;
Xaa at position 26 is His, Thr, Phe, Gly, Arg, Ala, or Trp;
Xaa at position 27 is Leu, Gly, Arg, Thr, Ser, or Ala;
Xaa at position 28 is Lys, Arg, Leu, Gln, Gly, Pro, Val or Trp;
Xaa at position 29 is Gln, Asn, Leu, Pro, Arg, or Val;
Xaa at position 30 is Pro, His, Thr, Gly, Asp, Gln, Ser, Leu, or Lys;
Xaa at position 31 is Pro, Asp, Gly, Ala, Arg, Leu, or Gln;

Xaa at position 32 is Leu, Val, Arg, Gln, Asn, Gly, Ala, or Glu;

Xaa at position 33 is Pro, Leu, Gln, Ala, Thr, or Glu;

Xaa at position 34 is Leu, Val, Gly, Ser, Lys, Glu, Gln, Thr, Arg, Ala, Phe, Ile or Met;

Xaa at position 35 is Leu, Ala, Gly, Asn, Pro, Gln, or Val;

Xaa at position 36 is Asp, Leu, or Val;

Xaa at position 37 is Phe, Ser, Pro, Trp, or Ile;

Xaa at position 38 is Asn, or Ala;

Xaa at position 40 is Leu, Trp, or Arg;

Xaa at position 41 is Asn, Cys, Arg, Leu, His, Met, or Pro;

Xaa at position 42 is Gly, Asp, Ser, Cys, Asn, Lys, Thr, Leu, Val, Glu, Phe, Tyr, Ile, Met or Ala;

Xaa at position 43 is Glu, Asn, Tyr, Leu, Phe, Asp, Ala, Cys, Gln, Arg, Thr, Gly or Ser;

Xaa at position 44 is Asp, Ser, Leu, Arg, Lys, Thr, Met, Trp, Glu, Asn, Gln, Ala or Pro;

Xaa at position 45 is Gln, Pro, Phe, Val, Met, Leu, Thr, Lys, Trp, Asp, Asn, Arg, Ser, Ala, Ile, Glu or His;

Xaa at position 46 is Asp, Phe, Ser, Thr, Cys, Glu, Asn, Gln, Lys, His, Ala, Tyr, Ile, Val or Gly;

Xaa at position 47 is Ile, Gly, Val, Ser, Arg, Pro, or His;

Xaa at position 48 is Leu, Ser, Cys, Arg, Ile, His, Phe, Glu, Lys, Thr, Ala, Met, Val or Asn;

Xaa at position 49 is Met, Arg, Ala, Gly, Pro, Asn, His, or Asp;

Xaa at position 50 is Glu, Leu, Thr, Asp, Tyr, Lys, Asn, Ser, Ala, Ile, Val, His, Phe, Met or Gln;

Xaa at position 51 is Asn, Arg, Met, Pro, Ser, Thr, or His;

Xaa at position 52 is Asn, His, Arg, Leu, Gly, Ser, or Thr;

Xaa at position 53 is Leu, Thr, Ala, Gly, Glu, Pro, Lys, Ser, or Met;

Xaa at position 54 is Arg, Asp, Ile, Ser, Val, Thr, Gln, Asn, Lys, His, Ala or Leu;

Xaa at position 55 is Arg, Thr, Val, Ser, Leu, or Gly;

Xaa at position 56 is Pro, Gly, Cys, Ser, Gln, Glu, Arg, His, Thr, Ala, Tyr, Phe, Leu, Val or Lys;

Xaa at position 57 is Asn or Gly;

Xaa at position 58 is Leu, Ser, Asp, Arg, Gln, Val, or Cys;

Xaa at position 59 is Glu, Tyr, His, Leu, Pro, or Arg;

Xaa at position 60 is Ala, Ser, Pro, Tyr, Asn, or Thr;
Xaa at position 61 is Phe, Asn, Glu, Pro, Lys, Arg, or Ser;
Xaa at position 62 is Asn, His, Val, Arg, Pro, Thr, Asp, or Ile;
Xaa at position 63 is Arg, Tyr, Trp, Lys, Ser, His, Pro, or Val;
Xaa at position 64 is Ala, Asn, Pro, Ser, or Lys;
Xaa at position 65 is Val, Thr, Pro, His, Leu, Phe, or Ser;
Xaa at position 66 is Lys, Ile, Arg, Val, Asn, Glu, or Ser;
Xaa at position 67 is Ser, Ala, Phe, Val, Gly, Asn, Ile, Pro, or His;
Xaa at position 68 is Leu, Val, Trp, Ser, Ile, Phe, Thr, or His;
Xaa at position 69 is Gln, Ala, Pro, Thr, Glu, Arg, Trp, Gly, or Leu;
Xaa at position 70 is Asn, Leu, Val, Trp, Pro, or Ala;
Xaa at position 71 is Ala, Met, Leu, Pro, Arg, Glu, Thr, Gln, Trp, or Asn;
Xaa at position 72 is Ser, Glu, Met, Ala, His, Asn, Arg, or Asp;
Xaa at position 73 is Ala, Glu, Asp, Leu, Ser, Gly, Thr, or Arg;
Xaa at position 74 is Ile, Met, Thr, Pro, Arg, Gly, Ala;
Xaa at position 75 is Glu, Lys, Gly, Asp, Pro, Trp, Arg, Ser, Gln, or Leu;
Xaa at position 76 is Ser, Val, Ala, Asn, Trp, Glu, Pro, Gly, or Asp;
Xaa at position 77 is Ile, Ser, Arg, Thr, or Leu;
Xaa at position 78 is Leu, Ala, Ser, Glu, Phe, Gly, or Arg;
Xaa at position 79 is Lys, Thr, Asn, Met, Arg, Ile, Gly, or Asp;
Xaa at position 80 is Asn, Trp, Val, Gly, Thr, Leu, Glu, or Arg;
Xaa at position 81 is Leu, Gln, Gly, Ala, Trp, Arg, Val, or Lys;
Xaa at position 82 is Leu, Gln, Lys, Trp, Arg, Asp, Glu, Asn, His, Thr, Ser, Ala, Tyr, Phe, Ile, Met or Val;
Xaa at position 83 is Pro, Ala, Thr, Trp, Arg, or Met;
Xaa at position 84 is Cys, Glu, Gly, Arg, Met, or Val;
Xaa at position 85 is Leu, Asn, Val, or Gln;
Xaa at position 86 is Pro, Cys, Arg, Ala, or Lys;
Xaa at position 87 is Leu, Ser, Trp, or Gly;
Xaa at position 88 is Ala, Lys, Arg, Val, or Trp;
Xaa at position 89 is Thr, Asp, Cys, Leu, Val, Glu, His, Asn, or Ser;

Xaa at position 90 is Ala, Pro, Ser, Thr, Gly, Asp, Ile, or Met;

Xaa at position 91 is Ala, Pro, Ser, Thr, Phe, Leu, Asp, or His;

Xaa at position 92 is Pro, Phe, Arg, Ser, Lys, His, Ala, Gly, Ile or Leu;

Xaa at position 93 is Thr, Asp, Ser, Asn, Pro, Ala, Leu, or Arg;

Xaa at position 94 is Arg, Ile, Ser, Glu, Leu, Val, Gln, Lys, His, Ala, or Pro;

Xaa at position 95 is His, Gln, Pro, Arg, Val, Leu, Gly, Thr, Asn, Lys, Ser, Ala, Trp, Phe, Ile, or Tyr;

Xaa at position 96 is Pro, Lys, Tyr, Gly, Ile, or Thr;

Xaa at position 97 is Ile, Val, Lys, Ala, or Asn;

Xaa at position 98 is His, Ile, Asn, Leu, Asp, Ala, Thr, Glu, Gln, Ser, Phe, Met, Val, Lys, Arg, Tyr or Pro;

Xaa at position 99 is Ile, Leu, Arg, Asp, Val, Pro, Gln, Gly, Ser, Phe, or His;

Xaa at position 100 is Lys, Tyr, Leu, His, Arg, Ile, Ser, Gln, or Pro;

Xaa at position 101 is Asp, Pro, Met, Lys, His, Thr, Val, Tyr, Glu, Asn, Ser, Ala, Gly, Ile, Leu, or Gln;

Xaa at position 102 is Gly, Leu, Glu, Lys, Ser, Tyr, or Pro;

Xaa at position 103 is Asp, or Ser;

Xaa at position 104 is Trp, Val, Cys, Tyr, Thr, Met, Pro, Leu, Gln, Lys, Ala, Phe, or Gly;

Xaa at position 105 is Asn, Pro, Ala, Phe, Ser, Trp, Gln, Tyr, Leu, Lys, Ile, Asp, or His;

Xaa at position 106 is Glu, Ser, Ala, Lys, Thr, Ile, Gly, or Pro;

Xaa at position 108 is Arg, Lys, Asp, Leu, Thr, Ile, Gln, His, Ser, Ala or Pro;

Xaa at position 109 is Arg, Thr, Pro, Glu, Tyr, Leu, Ser, or Gly;

Xaa at position 110 is Lys, Ala, Asn, Thr, Leu, Arg, Gln, His, Glu, Ser, or Trp;

Xaa at position 111 is Leu, Ile, Arg, Asp, or Met;

Xaa at position 112 is Thr, Val, Gln, Tyr, Glu, His, Ser, or Phe;

Xaa at position 113 is Phe, Ser, Cys, His, Gly, Trp, Tyr, Asp, Lys, Leu, Ile, Val or Asn;

Xaa at position 114 is Tyr, Cys, His, Ser, Trp, Arg, or Leu;

Xaa at position 115 is Leu, Asn, Val, Pro, Arg, Ala, His, Thr, Trp, or Met;

Xaa at position 116 is Lys, Leu, Pro, Thr, Met, Asp, Val, Glu, Arg, Trp, Ser, Asn, His, Ala, Tyr, Phe, Gln, or Ile;
Xaa at position 117 is Thr, Ser, Asn, Ile, Trp, Lys, or Pro;
Xaa at position 118 is Leu, Ser, Pro, Ala, Glu, Cys, Asp, or Tyr;
Xaa at position 119 is Glu, Ser, Lys, Pro, Leu, Thr, Tyr, or Arg;
Xaa at position 120 is Asn, Ala, Pro, Leu, His, Val, or Gln;
Xaa at position 121 is Ala, Ser, Ile, Asn, Pro, Lys, Asp, or Gly;
Xaa at position 122 is Gln, Ser, Met, Trp, Arg, Phe, Pro, His, Ile, Tyr, or Cys;
Xaa at position 123 is Ala, Met, Glu, His, Ser, Pro, Tyr, or Leu;

wherein from 4 to 44 of the amino acids designated by Xaa are different from the corresponding amino acids of native (1-133) human interleukin-3; wherein from 1 to 14 amino acids can be deleted from the N-terminus and/or from 1 to 15 amino acids can be deleted from the C-terminus of said interleukin-3 mutant polypeptide; and said interleukin-3 mutant polypeptide can additionally be immediately preceded by Methionine^{-1}, Alanine^{-1} or Methionine^{-2} Alanine^{-1}; and

(b) harvesting said cultured stem cells.

Additionally, the present invention encompasses methods of ex-vivo expansion of stem cells comprising the steps of (a) separating stem cells from a mixed population of cells; (b) culturing said separated stem cells with a growth medium comprising a human interleukin-3 mutant polypeptide; (c) harvesting said cultured cells.

The present invention includes methods for treatment of a patient having a hematopoietic disorder, comprising the steps of; (a) removing stem cells from said patient or a blood donor; (b) culturing said stem cells with a growth medium comprising a human interleukin-3 mutant polypeptide; (c) harvesting said

cultured cells; and (d) transplanting said cultured cells into said patient.

The present invention also includes methods for treatment of a patient having a hematopoietic disorder, comprising the steps of; (a) removing stem cells from said patient or a blood donor; (b) separating stem cells from a mixed population of cells; (c) culturing said separated stem cells with a growth medium comprising a human interleukin-3 mutant polypeptide; (d) harvesting said cultured cells; and (e) transplanting said cultured cells into said patient.

It is also envisioned that a patient could be given a hematopoietic growth factor, preferably a early acting factor, prior to removing stem cells for ex-vivo expansion to increase the number of early progenitors. It is also envisioned that a portion of the stem cells removed from a patient could be frozen and transplanted with the expanded stem cells to provide more early progenitors.

It is envisioned that the present invention includes methods of human gene therapy, comprising the steps of; (a) removing stem cells from a patient or blood donor; (b) culturing said stem cells with a selected growth medium comprising a human interleukin-3 mutant polypeptide; (c) introducing DNA into said cultured cells; (d) harvesting said transduced cells; and (e) transplanting said transduced cells into said patient.

It is also envisioned that the present invention includes methods of human gene therapy, comprising the steps of; (a) removing stem cells from a patient or blood donor; (b) separating said stem cells from a mixed population of cells; (c) culturing said separated stem cells with a selected growth medium comprising a human

interleukin-3 mutant polypeptide; (d) introducing DNA into said cultured cells; (e) harvesting said transduced cells; and (f) transplanting said transduced cells into said patient.

Brief Description of the Drawings

Figure 1 is the human IL-3 gene for E. coli expression (pMON5873), encoding the polypeptide sequence of natural (wild type) human IL-3 [SEQ ID NO:128], plus an initiator methionine, as expressed in E. coli, with the amino acids numbered from the N-terminus of the natural hIL-3.

Detailed Description of the Invention

The present invention relates to the use of muteins of human interleukin-3 (hIL-3) in which amino acid substitutions have been made at four or more positions in amino acid sequence of the polypeptide and to muteins which have substantially the same structure and substantially the same biological activity for the ex-vivo expansion of hematopoietic cells. Preferred muteins of the present invention are (15-125)hIL-3 deletion mutants which have deletions of amino acids 1 to 14 at the N-terminus and 126 to 133 at the C-terminus and which also have four or more amino acid substitutions in the polypeptide and muteins having substantially the same structure and substantially the same biological activity. Among the preferred muteins are those having twenty-six amino acid substitutions. As used herein human interleukin-3 corresponds to the amino acid sequence (1-133) as depicted in Figure 1 and (15-125) hIL-3 corresponds to the 15 to 125 amino acid sequence of the hIL-3 polypeptide. Naturally occurring variants of hIL-3 polypeptide amino acids are also

included in the present invention (for example, the allele in which proline rather than serine is at position 8 in the hIL-3 polypeptide sequence) as are variant hIL-3 molecules which are modified post-translationally (e.g. glycosylation).

Hematopoiesis requires a complex series of cellular events in which stem cells generate continuously into large populations of maturing cells in all major lineages. There are currently at least 20 known regulators with hematopoietic proliferative activity. Most of these proliferative regulators can stimulate one or another type of colony formation in vitro, the precise pattern of colony formation stimulated by each regulator is quite distinctive. No two regulators stimulate exactly the same pattern of colony formation, as evaluated by colony numbers or, more importantly, by the lineage and maturation pattern of the cells making up the developing colonies. Proliferative responses can most readily be analyzed in simplified in vitro culture systems. Three quite different parameters can be distinguished: alteration in colony size, alteration in colony numbers and cell lineage.

The mutant hIL-3 polypeptides of the present invention may also have methionine, alanine, or methionine-alanine residues inserted at the N-terminus.

"Mutant amino acid sequence," "mutant protein" or "mutant polypeptide" refers to a polypeptide having an amino acid sequence which varies from a native sequence or is encoded by a nucleotide sequence intentionally made variant from a native sequence. "Mutant protein," "variant protein" or "mutein" means a protein comprising a mutant amino acid sequence and includes polypeptides which differ from the amino acid sequence of native hIL-3 due to amino acid deletions, substitutions, or both. "Native sequence" refers to an amino acid or nucleic

acid sequence which is identical to a wild-type or native form of a gene or protein.

Human IL-3 can be characterized by its ability to stimulate colony formation by human hematopoietic progenitor cells. The colonies formed include erythroid, granulocyte, megakaryocyte, granulocytic macrophages and mixtures thereof. Human IL-3 has demonstrated an ability to restore bone marrow function and peripheral blood cell populations to therapeutically beneficial levels in studies performed initially in primates and subsequently in humans (Gillio, A. P., et al., *J. Clin. Invest.* **85**:1560 (1990); Ganser, A., et al., *Blood* **76**: 666 (1990); Falk, S., et al., *Hematopathology* **95**:355 (1991). Additional activities of hIL-3 include the ability to stimulate leukocyte migration and chemotaxis; the ability to prime human leukocytes to produce high levels of inflammatory mediators like leukotrienes and histamine; the ability to induce cell surface expression of molecules needed for leukocyte adhesion; and the ability to trigger dermal inflammatory responses and fever. Other IL-3-like properties are the interaction with early multipotential stem cells, the sustaining of the growth of pluripotent precursor cells, the ability to stimulate chronic myelogenous leukemia (CML) cell proliferation, the stimulation of proliferation of mast cells, the ability to support the growth of various factor-dependent cell lines, and the ability to trigger immature bone marrow cell progenitors. Other biological properties of IL-3 have been disclosed in the art. Many or all of these biological activities of hIL-3 involve signal transduction and high affinity receptor binding.

Biological activity of hIL-3 and hIL-3 varinats of the present invention is determined by DNA synthesis by human acute myelogenous leukemia cells (AML). The factor-dependent cell line AML 193 was adapted for use in testing biological activity. The biological activity

of hIL-3 and hIL-3 chimera proteins of the present invention is also determined by counting the colony forming units in a bone marrow assay.

Other in vitro cell based assays may also be useful to determine the activity of the chimera molecules depending on the hematopoietic growth factors that comprise the chimera. The following are examples of other useful assays.

TF-1 proliferation assay: The TF-1 cell line was derived from bone marrow of a patient with erythroleukemia (Kitamura et al., *J. Cell Physiol.* **140**:323-334, 1989). TF-1 cells respond to IL-3, GM-CSF, EPO and IL-5.

Compounds of this invention are preferably made by genetic engineering techniques now standard in the art United States Patent 4,935,233 and Sambrook et al., "Molecular Cloning. A Laboratory Manual", Cold Spring Harbor Laboratory, 1989. One method of creating the preferred hIL-3 (15-125) mutant genes is cassette mutagenesis (Wells, et al., *Gene*, **34**:315-323, 1985) in which a portion of the coding sequence of hIL-3 in a plasmid is replaced with synthetic oligonucleotides that encode the desired amino acid substitutions in a portion of the gene between two restriction sites. In a similar manner amino acid substitutions could be made in the full-length hIL-3 gene, or genes encoding variants of hIL-3 in which from 1 to 14 amino acids have been deleted from the N-terminus and/or from 1 to 15 amino acids have been deleted from the C-terminus. When properly assembled these oligonucleotides would encode hIL-3 variants with the desired amino acid substitutions and/or deletions from the N-terminus and/or C-terminus. These and other mutations could be created by those skilled in the art by other mutagenesis methods including; oligonucleotide-directed mutagenesis (Zoller and Smith, *Nucleic Acid Research*, **10**:6487-6500, 1982; Zoller and Smith, *Methods in Enzymology*, **100**:468-500,

1983; Zoller and Smith, *DNA*, **3**: 479, 1984 Smith M., *Ann. Rev. Genet.*, **19**:423-462, 1985; Kunkel, *Proc. Natl. Acad. Sci. USA*, **82**:488-492, 1985, Taylor, et al., *Nucl. Acids Res.* **13**:8764-8785 (1985), Deng and Nickoloff, *Anal-Biochem* **200**:81-88, 1992) or polymerase chain reaction (PCR) techniques (Saiki, *Science* **230**:1350-1354, 1985).

Additional details about recombinant techniques for construction of DNA sequences that encode the IL-3 variants, plasmid DNA vectors for use in the expression of these novel chimera molecules, methods for producing the IL-3 variants in bacterial cells, mammalian cells, or insect cells can be found in WO 94/12638. It is understood that the IL-3 variants of the present invention, used for the ex-vivo expansion of hematopoietic cells, can be made by other methods known to those skilled in the art.

<u>Interpretation of activity of single amino acid mutants in IL-3 (15-125)</u>

As illustrated in Tables 6 and 9 of WO 94/12638, there are certain positions in the IL-3 (15-125) molecule which are intolerant of substitutions, in that most or all substitutions at these positions resulted in a considerable decrease in bioactivity. There are two likely classes of such "down-mutations": mutations that affect overall protein structure, and mutations that interfere directly with the interaction between the IL-3 molecule and its receptor. Mutations affecting the three-dimensional structure of the protein will generally lie in the interior of the protein, while mutations affecting receptor binding will generally lie on the surface of the protein. Although the three-dimensional structure of IL-3 is unknown, there are simple algorithms which can aid in the prediction of the structure. One such algorithm is the use of "helical wheels" (Kaiser, E.T. & Kezdy, F.J., *Science* **223**:249-

255, 1984). In this method, the presence of alpha helical protein structures can be predicted by virtue of their amphipathic nature. Helices in globular proteins commonly have an exposed hydrophilic side and a buried hydrophobic side. As a broad generalization, in globular proteins, hydrophobic residues are present in the interior of the protein, and hydrophilic residues are present on the surface. By displaying the amino acid sequence of a protein on such a "helical wheel" it is possible to derive a model for which amino acids in alpha helices are exposed and which are buried in the core of the protein. Such an analysis of the IL-3 (15-125) molecule predicts that the following helical residues are buried in the core:

M19, I20, I23, I24, L27, L58, F61, A64, L68, A71, I74, I77, L78, L81, W104, F107, L111, Y114, L115, L118.

In addition, cysteine residues at positions 16 and 84 are linked by a disulfide bond, which is important for the overall structure or "folding" of the protein. Finally, mutations which result in a major disruption of the protein structure may be expressed at low level in the secretion system used in our study, for a variety of reasons: either because the mis-folded protein is poorly recognized by the secretion machinery of the cell; because mis-folding of the protein results in aggregation, and hence the protein cannot be readily extracted from the cells; or because the mis-folded protein is more susceptible to degradation by cellular proteases. Hence, a block in secretion may indicate which positions in the IL-3 molecule which are important for maintenance of correct protein structure.

In order to retain the activity of a variant of IL-3, it is necessary to retain both the structural integrity of the protein, and retain the specific residues important for receptor contact. Hence it is

possible to define specific amino acid residues in IL-3 (15-125) which must be retained in order to preserve biological activity.

Residues predicted to be important for interaction with the receptor: D21, E22, E43, D44, L48, R54, R94, D103, K110, F113.

Residues predicted to be structurally important: C16, L58, F61, A64, I74, L78, L81, C84, P86, P92, P96, F107, L111, L115, L118.

One aspect of the present invention provides a novel hematopoietic factors for selective ex-vivo expansion of stem cells. The term "stem cell" refers to the totipiotent hematopoietic stem cells as well as early precursors and progenitor cells which can be isolated from bone marrow, spleen or peripheral blood. The term "expanding" refers to the differentiation and proliferation of the cells. The present invention provides a method for selective ex-vivo expansion of stem cells, comprising the steps of; (a) separating stem cells from a mixed population of cells, (b) culturing said separated stem cells with a selected media which contains a mutant interleukin-3 polypeptide and (c) harvesting said cultured stems cells.

Stem cells as well as committed progenitor cells destined to become neutrophils, erythrocytes, platelets. etc., may be distinguished from most other cells by the presence or absence of particular progenitor marker antigens, such as CD34, that are present on the surface of these cells and/or by morphological characteristics. The phenotype for a highly enriched human stem cell fraction is reported as CD34+, Thy-1+ and lin-, but it is to be understood that the present invention is not limited to the expansion of this stem cell population. The CD34+ enriched human stem cell fraction can be separated by a number of reported methods, including

affinity columns or beads, magnetic beads or flow cytometry using antibodies directed to surface antigens such as the CD34+. Further, physical separation methods such as counterflow elutriation may be used to enrich hematopoietic progenitors. The CD34+ progenitors are heterogeneous, and may be divided into several subpopulations characterized by the presence or absence of coexpression of different lineage associated cell surface associated molecules. The most immature progenitor cells do not express any known lineage-associated markers, such as HLA-DR or CD38, but they may express CD90(thy-1). Other surface antigens such as CD33, CD38, CD41, CD71, HLA-DR or c-kit can also be used to selectively isolate hematopoietic progenitors. The separated cells can be incubated in selected medium in a culture flask, sterile bag or in hollow fibers. Various hematopoietic growth factors may be utilized in order to selectively expand cells. Representative factors that have been utilized for ex-vivo expansion of bone marrow include, c-kit ligand, IL-3, G-CSF, GM-CSF, IL-1, IL-6, IL-11, flt-3 ligand or combinations thereof. The proliferation of the stem cells can be monitored by enumerating the number of stem cells and other cells, by standard techniques (e.g. hemacytometer, CFU, LTCIC) or by flow cytometry prior and subsequent to incubation.

Several methods for ex-vivo expansion of stem cells have been reported utilizing a number of selection methods and expansion using various hematopoietic growth factors including c-kit ligand (Brandt et al., *Blood* **83**:1507-1514 (1994), McKenna et al., Blood 86:3413-3420 (1995), IL-3 (Brandt et al., *Blood* **83**:1507-1514 (1994), Sato et al., *Blood* **82**:3600-3609 (1993), G-CSF (Sato et al., *Blood* **82**:3600-3609 (1993), GM-CSF (Sato et al., *Blood* **82**:3600-3609 (1993), IL-1 (Muench et al., *Blood* **81**:3463-3473 (1993), IL-6 (Sato et al., *Blood* **82**:3600-3609 (1993), IL-11 (Lemoli et al., *Exp. Hem.* **21**:1668-1672 (1993), Sato et al., *Blood* **82**:3600-3609 (1993),

flt-3 ligand (McKenna et al., Blood 86:3413-3420 (1995) and/or combinations thereof (Brandt et al., *Blood* **83**:1507-1514 (1994), Haylock et al., *Blood* **80**:1405-1412 (1992), Koller et al., *Biotechnology* **11**:358-363 (1993), (Lemoli et al., *Exp. Hem.* **21**:1668-1672 (1993), McKenna et al., Blood 86:3413-3420 (1995), Muench et al., *Blood* **81**:3463-3473 (1993), Patchen et al., *Biotherapy* **7**:13-26 (1994), Sato et al., *Blood* **82**:3600-3609 (1993), Smith et al., *Exp. Hem.* **21**:870-877 (1993), Steen et al., *Stem Cells* **12**:214-224 (1994), Tsujino et al., *Exp. Hem.* **21**:1379-1386 (1993). Among the individual hematopoietic growth factors, hIL-3 has been shown to be one of the most potent in expanding peripheral blood CD34+ cells (Sato et al., *Blood* **82**:3600-3609 (1993), Kobayashi et al., *Blood* **73**:1836-1841 (1989). However, no single factor has been shown to be as effective as the combination of multiple factors. The present invention provides methods for ex vivo expansion that utilize molecules that are more effective than IL-3 alone.

Another projected clinical use of growth factors has been in the in vitro activation of hematopoietic progenitors and stem cells for gene therapy. Due to the long life-span of hematopoietic progenitor cells and the distribution of their daughter cells throughout the entire body, hematopoietic progenitor cells are good candidates for ex vivo gene transfection. In order to have the gene of interest incorporated into the genome of the hematopoietic progenitor or stem cell one needs to stimulate cell division and DNA replication. Hematopoietic stem cells cycle at a very low frequency which means that growth factors may be useful to promote gene transduction and thereby enhance the clinical prospects for gene therapy. Potential applications of gene therapy (review Crystal, Science **270**:404-410 (1995) include; 1) the treatment of many congenital metabolic disorders and immunodifiencies (Kay and Woo, *Trends Genet.* **10**:253-257 (1994), 2) neurological disorders

(Freedmann, *Trends Genet.* **10**:210-214 (1994), 3) cancer (Culver and Blaese, *Trends Genet.* **10**:174-178 (1994) and 4) infectious diseases (Gilboa and Smith, *Trends Genet.* **10**:139-144 (1994). Due to the long life-span of hematopoietic progenitor cells and the distribution of their daughter cells throughout the entire body, hematopoietic progenitor cells are good candidates for ex vivo gene transfection include the treatment of many congenital metabolic disorders and immunodifiencies (Kay and Woo, *Trends Genet.* **10**:253-257 (1994) neurological disorders (Freedmann, *Trends Genet.* **10**:210-214 (1994), cancer (Culver and Blaese, *Trends Genet.* **10**:174-178 (1994) and infectious diseases (Gilboa and Smith, *Trends Genet.* **10**:139-144 (1994).

There are a variety of methods, known to those with skill in the art, for introducing genetic material into a host cell. A number of vectors, both viral and non-viral have been developed for transferring therapeutic genes into primary cells. Viral based vectors include; 1) replication-deficient recombinant retrovirus (Boris-Lawrie and Temin, *Curr. Opin. Genet. Dev.* **3**:102-109 (1993), Boris-Lawrie and Temin, *Annal. New York Acad. Sci.* **716**:59-71 (1994), Miller, *Current Top. Microbiol. Immunol.* **158**:1-24 (1992) and replication-deficient recombinant adenovirus (Berkner, *BioTechniques* **6**:616-629 (1988), Berkner, *Current Top. Microbiol. Immunol.* **158**:39-66 (1992), Brody and Crystal, *Annal. New York Acad. Sci.* **716**:90-103 (1994). Non-viral based vectors include protein/DNA complexes (Cristiano et al., *PNAS USA*. **90**:2122-2126 (1993), Curiel et al., *PNAS USA* **88**:8850-8854 (1991), Curiel, *Annal. New York Acad. Sci.* **716**:36-58 (1994), electroporation and liposome mediated delivery such as cationic liposomes (Farhood et al., *Annal. New York Acad. Sci.* **716**:23-35 (1994).

Hematopoietic cells that have been expanded ex-vivo using hIL-3 variants of the present invention may be useful in the treatment of diseases characterized by a

decreased levels of either myeloid, erythroid, lymphoid, or megakaryocyte cells of the hematopoietic system or combinations thereof. In addition, they may be used to activate mature myeloid and/or lymphoid cells. Among conditions susceptible to treatment with the hematopoietic cells that have been expanded ex-vivo using hIL-3 variants of the present invention is leukopenia, a reduction in the number of circulating leukocytes (white cells) in the peripheral blood. Leukopenia may be induced by exposure to certain viruses or to radiation. It is often a side effect of various forms of cancer therapy, e.g., exposure to chemotherapeutic drugs and of infection or hemorrhage. Therapeutic treatment of leukopenia with these expanded hematopoietic cells may avoid undesirable side effects caused by treatment with current drugs.

The expanded hematopoietic cells of the present invention may be useful in the treatment of neutropenia and, for example, in the treatment of such conditions as aplastic anemia, cyclic neutropenia, idiopathic neutropenia, Chediak-Higashi syndrome, systemic lupus erythematosus (SLE), leukemia, myelodysplastic syndrome and myelofibrosis.

Many drugs may cause bone marrow suppression or hematopoietic deficiencies. Examples of such drugs are AZT, DDI, alkylating agents and anti-metabolites used in chemotherapy, antibiotics such as chloramphenicol, penicillin and sulfa drugs, phenothiazones, tranquilizers such as meprobamate, and diuretics. The expanded hematopoietic cells of the present invention may be useful in preventing or treating the bone marrow suppression or hematopoietic deficiencies which often occur in patients treated with these drugs.

Hematopoietic deficiencies may also occur as a result of viral, microbial or parasitic infections and as a result of treatment for renal disease or renal failure, e.g., dialysis. The expanded hematopoietic cells of the present invention may be useful in treating

such hematopoietic deficiency.

Various immunodeficiencies e.g., in T and/or B lymphocytes, or immune disorders, e.g., rheumatoid arthritis, may also be beneficially affected by expanded hematopoietic cells of the present invention. Immunodeficiencies may be the result of viral infections e.g. HTLVI, HTLVII, HTLVIII, severe exposure to radiation, cancer therapy or the result of other medical treatment. The expanded hematopoietic cells of the present invention may also be employedn in the treatment of other blood cell deficiencies, including thrombocytopenia or anemia.

The dosage regimen involved in ex-vivo expansion of hematopoietic cells and methods for treating the above-described conditions will be determined by the attending physician considering various factors which modify the action of drugs, e.g. the condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. Generally, a daily regimen may be in the range of 1ng/ml to 100ng/mL of non-glycosylated IL-3 protein per mL of culture medium. This dosage regimen is referenced to a standard level of biological activity which recognizes that native IL-3 generally possesses an EC_{50} at or about 10 picoMolar to 100 picoMolar in the AML proliferation assay described herein. Therefore, dosages would be adjusted relative to the activity of a given mutein vs. the activity of native (reference) IL-3 and it would not be unreasonable to note that dosage regimens may include doses as low as 0.1 ng and as high as 1 milligram per mL of culture medium. In addition, there may exist specific circumstances where dosages of IL-3 mutein would be adjusted higher or lower These include co-administration with other hematopoietic growth factors; co-administration with chemotherapeutic drugs and/or radiation; the use of glycosylated IL-3 mutein; and various patient-related issues mentioned earlier in this

section.

EXAMPLE 1

AML Proliferation Assay for Bioactive Human Interleukin-3

hIL-3 SANDWICH ELISA

IL-3 protein concentrations can be determined using a sandwich ELISA based on an affinity purified polyclonal goat anti-rhIL-3. Microtiter plates (Dynatech Immulon II) were coated with 150 µl goat-anti-rhIL-3 at a concentration of approximately 1 µg/ml in 100 mM NaHCO3, pH 8.2. Plates were incubated overnight at room temperature in a chamber maintaining 100% humidity. Wells were emptied and the remaining reactive sites on the plate were blocked with 200 µl of solution containing 10 mM PBS, 3% BSA and 0.05% Tween 20, pH 7.4 for 1 hour at 37° C and 100% humidity. Wells were emptied and washed 4X with 150 mM NaCl containing 0.05% Tween 20 (wash buffer). Each well then received 150 µl of dilution buffer (10 mM PBS containing 0.1% BSA, 0.01% Tween 20, pH 7.4), containing rhIL-3 standard, control, sample or dilution buffer alone. A standard curve was prepared with concentrations ranging from 0.125 ng/ml to 5 ng/ml using a stock solution of rhIL-3 (concentration determined by amino acid composition analysis). Plates were incubated 2.5 hours at 37° C and 100% humidity. Wells were emptied and each plate was washed 4X with wash buffer. Each well then received 150 µl of an optimal dilution (as determined in a checkerboard assay format) of goat anti-rhIL-3 conjugated to horseradish peroxidase. Plates were incubated 1.5 hours at 37° C and 100% humidity. Wells were emptied and each plate was washed 4X with wash buffer. Each well then received 150 ul of ABTS substrate solution (Kirkegaard and Perry). Plates were incubated at room temperature until the color of the

standard wells containing 5 ng/ml rhIL-3 had developed enough to yield an absorbance between 0.5-1.0 when read at a test wavelength of 410 nm and a reference wavelength of 570 nm on a Dynatech microtiter plate reader. Concentrations of immunoreactive rhIL-3 in unknown samples were calculated from the standard curve using software supplied with the plate reader.

AML Proliferation Assay for Bioactive Human Interleukin-3

The factor-dependent cell line AML 193 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). This cell line, established from a patient with acute myelogenous leukemia, is a growth factor dependent cell line which displayed enhanced growth in GM/CSF supplemented medium (Lange, B., et al., *Blood* **70**:192, 1987; Valtieri, M., et al., *J. Immunol* **138**:4042, 1987). The ability of AML 193 cells to proliferate in the presence of human IL-3 has also been documented. (Santoli, D., et al., *J. Immunol* **139**:348, 1987). A cell line variant was used, AML 193 1.3, which was adapted for long term growth in IL-3 by washing out the growth factors and starving the cytokine dependent AML 193 cells for growth factors for 24 hours. The cells were then replated at $1x10^5$ cells/well in a 24 well plate in media containing 100 U/ml IL-3. It took approximately 2 months for the cells to grow rapidly in IL-3. These cells were maintained as AML 193 1.3 thereafter by supplementing tissue culture medium (see below) with human IL-3.

AML 193 1.3 cells were washed 6 times in cold Hanks balanced salt solution (HBSS, Gibco, Grand Island, NY) by centrifuging cell suspensions at 250 x g for 10 minutes followed by decantation of supernatant. Pelleted cells were resuspended in HBSS and the procedure was repeated until six wash cycles were completed. Cells washed six times by this procedure

were resuspended in tissue culture medium at a density ranging from 2 x 10^5 to 5 x 10^5 viable cells/ml. This medium was prepared by supplementing Iscove's modified Dulbecco's Medium (IMDM, Hazleton, Lenexa, KS) with albumin, transferrin, lipids and 2-mercaptoethanol. Bovine albumin (Boehringer-Mannheim, Indianapolis, IN) was added at 500 μg/ml; human transferrin (Boehringer-Mannheim, Indianapolis, IN) was added at 100 μg/ml; soybean lipid (Boehringer-Mannheim, Indianapolis, IN) was added at 50 μg/ml; and 2-mercaptoethanol (Sigma, St. Louis, MO) was added at 5 x 10^{-5} M.

Serial dilutions of human interleukin-3 or human interleukin-3 variant protein (hIL-3 mutein) were made in triplicate series in tissue culture medium supplemented as stated above in 96 well Costar 3596 tissue culture plates. Each well contained 50 μl of medium containing interleukin-3 or interleukin-3 variant protein once serial dilutions were completed. Control wells contained tissue culture medium alone (negative control). AML 193 1.3 cell suspensions prepared as above were added to each well by pipetting 50 μl (2.5 x 10^4 cells) into each well. Tissue culture plates were incubated at 37°C with 5% CO_2 in humidified air for 3 days. On day 3, 0.5 μCi ^{3}H-thymidine (2 Ci/mM, New England Nuclear, Boston, MA) was added in 50 μl of tissue culture medium. Cultures were incubated at 37°C with 5% CO_2 in humidified air for 18-24 hours. Cellular DNA was harvested onto glass filter mats (Pharmacia LKB, Gaithersburg, MD) using a TOMTEC cell harvester (TOMTEC, Orange, CT) which utilized a water wash cycle followed by a 70% ethanol wash cycle. Filter mats were allowed to air dry and then placed into sample bags to which scintillation fluid (Scintiverse II, Fisher Scientific, St. Louis, MO or BetaPlate Scintillation Fluid, Pharmacia LKB, Gaithersburg, MD) was added. Beta emissions of samples from individual tissue culture wells were counted in a LKB Betaplate model 1205 scintillation counter (Pharmacia LKB, Gaithersburg, MD)

and data was expressed as counts per minute of ^{3}H-thymidine incorporated into cells from each tissue culture well. Activity of each human interleukin-3 preparation or human interleukin-3 variant preparation was quantitated by measuring cell proliferation (^{3}H-thymidine incorporation) induced by graded concentrations of interleukin-3 or interleukin-3 variant. Typically, concentration ranges from 0.05 pM - 10^{5} pM are quantitated in these assays. Activity is determined by measuring the dose of interleukin-3 or interleukin-3 variant which provides 50% of maximal proliferation [EC_{50} = 0.5 x (maximum average counts per minute of ^{3}H-thymidine incorporated per well among triplicate cultures of all concentrations of interleukin-3 tested - background proliferation measured by ^{3}H-thymidine incorporation observed in triplicate cultures lacking interleukin-3]. This EC_{50} value is also equivalent to 1 unit of bioactivity. Every assay was performed with native interleukin-3 as a reference standard so that relative activity levels could be assigned.

Relative biological activities of IL-3 muteins of the present invention are shown in Table 1. The Relative Biological Activity of IL-3 mutants is calculated by dividing the EC_{50} of (1-133) hIL-3 by the EC_{50} of the mutant. The Relative Biological Activity may be the average of replicate assays.

TABLE 1

BIOLOGICAL ACTIVITY OF IL-3 MUTEINS

Plasmid Code	Polypeptide Structure	Relative* Biological Activity
Reference (1-133)hIL-3		1
pMON13298	SEQ ID NO. 82	3
pMON13299	SEQ ID NO. 83	2
pMON13300	SEQ ID NO. 84	3
pMON13301	SEQ ID NO. 85	2
pMON13302	SEQ ID NO. 86	1.2
pMON13303	SEQ ID NO. 87	0.6
pMON13287	SEQ ID NO. 88	26
pMON13288	SEQ ID NO. 89	24
pMON13289	SEQ ID NO. 90	13
pMON13290	SEQ ID NO. 91	20
pMON13292	SEQ ID NO. 92	6
pMON13294	SEQ ID NO. 93	3
pMON13295	SEQ ID NO. 94	3
pMON13312	SEQ ID NO. 95	4
pMON13313	SEQ ID NO. 96	8
pMON13285	SEQ ID NO. 259	32
pMON13286	SEQ ID NO. 260	8
pMON13325	SEQ ID NO. 261	8
pMON13326	SEQ ID NO. 262	25
pMON13330	SEQ ID NO. 263	19
pMON13329	SEQ ID NO. 406	10
pMON13364	SEQ ID NO. 117	13
pMON13475	SEQ ID NO. 280	7
pMON13366	SEQ ID NO. 281	38
pMON13367	SEQ ID NO. 282	36
pMON13368	SEQ ID NO. 278	1.6
pMON13369	SEQ ID NO. 283	10
pMON13370	SEQ ID NO. 284	6
pMON13373	SEQ ID NO. 285	12
pMON13374	SEQ ID NO. 286	6
pMON13375	SEQ ID NO. 287	14
pMON13376	SEQ ID NO. 288	0.4
pMON13377	SEQ ID NO. 289	0.4
pMON13379	SEQ ID NO. 291	0.9
pMON13380	SEQ ID NO. 279	0.05
pMON13381	SEQ ID NO. 293	10
pMON13382	SEQ ID NO. 313	38
pMON13383	SEQ ID NO. 294	0.5
pMON13384	SEQ ID NO. 295	0.25
pMON13385	SEQ ID NO. 292	1

TABLE 1 (cont'd)

BIOLOGICAL ACTIVITY OF IL-3 MUTEINS

Plasmid Code	Polypeptide Structure	Relative* Biological Activity
pMON13387	SEQ ID NO. 308	32
pMON13388	SEQ ID NO. 296	23
pMON13389	SEQ ID NO. 297	10
pMON13391	SEQ ID NO. 298	30
pMON13392	SEQ ID NO. 299	17
pMON13393	SEQ ID NO. 300	32
pMON13394	SEQ ID NO. 301	20
pMON13395	SEQ ID NO. 302	11
pMON13396	SEQ ID NO. 303	20
pMON13397	SEQ ID NO. 304	16
pMON13398	SEQ ID NO. 305	36
pMON13399	SEQ ID NO. 306	18
pMON13404	SEQ ID NO. 307	1.3
pMON13417	SEQ ID NO. 310	24
pMON13420	SEQ ID NO. 311	19
pMON13421	SEQ ID NO. 312	0.5
pMON13432	SEQ ID NO. 313	10
pMON13400	SEQ ID NO. 317	0.09
pMON13402	SEQ ID NO. 318	20
pMON13403	SEQ ID NO. 321	0.03
pMON13405	SEQ ID NO. 267	9
pMON13406	SEQ ID NO. 264	5
pMON13407	SEQ ID NO. 266	16
pMON13408	SEQ ID NO. 269	7
pMON13409	SEQ ID NO. 270	15
pMON13410	SEQ ID NO. 271	0.4
pMON13411	SEQ ID NO. 322	1.2
pMON13412	SEQ ID NO. 323	0.5
pMON13413	SEQ ID NO. 324	0.6
pMON13414	SEQ ID NO. 265	4
pMON13415	SEQ ID NO. 268	4
pMON13418	SEQ ID NO. 326	0.5
pMON13419	SEQ ID NO. 325	0.015
pMON13422	SEQ ID NO. 272	0.4
pMON13423	SEQ ID NO. 273	0.4
pMON13424	SEQ ID NO. 274	3
pMON13425	SEQ ID NO. 275	6
pMON13426	SEQ ID NO. 276	>0.0003
pMON13429	SEQ ID NO. 277	>0.0002
pMON13440	SEQ ID NO. 319	9

TABLE 1 (cont'd)

BIOLOGICAL ACTIVITY OF IL-3 MUTEINS

Plasmid Code	Polypeptide Structure	Relative* Biological Activity
pMON13451	SEQ ID NO. 320	0.1
pMON13459	SEQ ID NO. 328	0.003
pMON13416	SEQ ID NO. 309	19.9
pMON13428	SEQ ID NO. 327	0.008
pMON13467	SEQ ID NO. 329	0.16
pMON13446	SEQ ID NO. 315	21.5
pMON13390	SEQ ID NO. 316	20

* The Relative Biological Activity of IL-3 mutants is calculated by dividing the EC_{50} of (1-133) hIL-3 by the EC_{50} of the mutant.

EXAMPLE 2

Determination of bioactivity of IL-3 variants and other hematopoietic growth factors in the Methylcellulose Assay

Methylcellulose Assay

This assay provides a reasonable approximation of the growth activity of colony stimulating factors to stimulate normal bone marrow cells to produce different types of hematopoietic colonies in vitro (Bradley et al., *Aust. Exp. Biol. Med. Sci.* **44**:287-300 1966; Pluznik et al., *J Cell Comp Physiol* **66**:319-324 1965).

Methods

Approximately 30 ml of fresh, normal, healthy bone marrow aspirate are obtained from individuals. Under sterile conditions samples are diluted 1:5

with a 1X PBS (#14040.059 Life Technologies, Gaithersburg, MD.) solution in a 50 ml conical tube (#25339-50 Corning, Corning MD). Ficoll (Histopaque-1077 Sigma H-8889) is layered under the diluted sample and centrifuged, 300 x g for 30 min. The mononuclear cell band is removed and washed two times in 1X PBS and once with 1% BSA PBS (CellPro Co., Bothel, WA). Mononuclear cells are counted and CD34+ cells are selected using the Ceprate LC (CD34) Kit (CellPro Co., Bothel, WA) column. This fractionation is performed since all stem and progenitor cells within the bone marrow display CD34 surface antigen. Alternatively whole bone marrow or peripheral blood may be used.

Cultures are set up in triplicate wells with a final volume of 0.1 ml in 48 well tissue culture plates (#3548 CoStar, Cambridge, MA). Culture medium is purchased from Terry Fox Labs. (HCC-4330 medium (Terry Fox Labs, Vancouver, B.C., Canada)). 600-1000 CD34+ cells are added per well. Native IL-3 and IL-3 variants are added to give final concentrations ranging from .001nM-10nM. G-CSF and GM-CSF and C-Kit ligand are added at a final concentration of 0.1nM. Native IL-3 and IL-3 variants are supplied in house. C-Kit Ligand (#255-CS), G-CSF (#214-CS) and GM-CSF (#215-GM) are purchased from R&D Systems (Minneapolis, MN). Cultures are resuspended using an Eppendorf repeater and 0.1 ml is dispensed per well. Control (baseline response) cultures received no colony stimulating factors. Positive control cultures received conditioned media (PHA stimulated human cells:Terry Fox Lab. H2400). Cultures are incubated at 37°C, 5% CO2 in humidified air. Hematopoietic colonies which are defined as greater than 50 cells are counted on the day of peak response (days 10-11) using a Nikon inverted phase

microscope with a 40x objective combination. Groups of cells containing fewer than 50 cells are referred to as clusters. Alternatively colonies can be identified by spreading the colonies on a slide and stained or they can be picked, resuspended and spun onto cytospin slides for staining.

Example 3

Ex vivo expansion of CD34+ cells from peripheral blood using chimera molecules pMON13416 compared with human IL-3

Flow Cytometry Evaluation

The percentage of CD34+ cells in the thawed peripheral blood cell population was determined by flow cytometry. Cells were removed from the selected cell population and placed into two centrifuge tube and washed once in 9/1% albumin Phosphate buffer (PAB). Twenty microliters of anti-CD34 monoclonal antibody (8G12-FITC) or mouse monoclonal antibody IgG-FITC control was added to the tube. The tubes were incubated for 15 minutes on ice. The cells were washed once with PAB and resuspended in approximately 0.5 mL PAB. Propidium iodide (2 ug/mL) was added to each tube just prior to the analysis on the FACSort or FACScan. Selected cells that contain greater than 80% CD34+ cells were used to initiate the cultures.

On day 12, cultures were harvested and evaluated with CD41A-FITC (a megakaryocyte marker), CD15-FITC and CD11b-PE (early to late neutrophil marker) and CD34 by flow cytometry, using the same processes of preparation and analysis as described above.

Colony Assay Evaluation

Colony assay evaluation was performed on day 0 with 500-1000 selected CD34+ cells per dish and again on day 12 of culture with 5,000-10,000 cultured cells per dish. The cells were added to a colony assay culture tube containing 3 mL of Terry Fox Iscove's based methylcellulose and the following growth factors: 20 ng/mL SCF, 10 U/mL EPO, 300 U/mL GM-CSF, 300 U/mL G-CSF, 30 U/mL IL3, 50ng/mL of IL-3 variants and 40 ng/mL IL6. Two 35mm tissue culture dishes containing 1 mL were set up. All dishes were incubated at 37°C, 5% carbon dioxide, 5% oxygen and high humidity for 13-15 days. The dishes were scored for myeloid (CFU-GM), erythroid (BFU-E) or mixed myeloid and erythroid colonies (CFU-mix) using a Nikon SMZU stereoscope.

Cell Morphology Evaluation

On day 12 of culture cells were analyzed for cell morphology after Wright-Giemsa staining. Cultured cells were cytocentrifuged onto slides at 1000 rpm for 4 minutes. Each slide contained approximately 10000-20000 cells. Slides were allowed to air dry before staining with 0.5 mL Wright-Giemsa for 1 minutes and 0.5 mL tap water for 1-2 minutes. Slides were cover-slipped and evaluated using a Microstar light microscope. A differential cell count of neutrophils, megakaryocytes and other blood cells was performed.

RESULTS

CD34+ Selection

Studies were performed on CD34+ cells selected using the Isolex™ 300 magnetic Cell Separator from

apheresis products from normal donors mobilized with G-CSF. The selected cells were stored in X-VIVO 10 +12.5%HSA containing 10% DMSO in liquid nitrogen until required. Cultures were initiated as described in the methods section.

Proliferation Index Of Cultures At Day 12

The proliferation index of cultures was calculated by diving the cell concentration at day 5-7 by $5X10^4$ and then multiplying it by the cell concentration at day 12 divided by $1X10^5$. A summary of the proliferation index obtained from these CD34+ cell cultures is shown in Table 5.

Flow Cytometry Evaluation Of Neutrophil Precursors

The percentage of neutrophil precursors in the CD34+ cell cultures at day 12 was assessed by flow cytometry using the CD15 marker for early to late neutrophil precursors and the CD11b marker found on late neutrophil precursors determined is shown in Table 2.

Flow Cytometry Evaluation Of Megakaryocytes

The percentage of Mks in the CD34+ cell cultures was assessed by flow cytometry using the CD41a marker for megakaryocytes. The percentage of Mks observed in the CD34+ cell cultures is shown in Table 2.

Flow Cytometry Evaluation Of CD34+ Cells

The percentage of CD34+ cells present in the cultures at day 12 was determined by flow cytometry. The percentage of CD34+ cells still remaining in the cultures at day 12 ranged from

0.103-19.3%, with no significant difference or patterns observed with the different growth factor combinations.

Total Number Of Megakaryocytes Generated In Culture

The total number of megakaryocytes present in each culture is calculated by multiplying the total number of cells at day 12 by the percentage of CD15+ cells and is shown in Table 2.

Colony Forming Unit Granulocyte-Macrophage (CFU-GM) Index

CFU-GM index is calculate`by dividing the total number of GM-colonies obtained at day 12 by the number of GM-colonies obtained at day 0. A CFU-GM index of 1 indicates that the number of colonies at day 12 is equivalent to the number of colonies at the start of the culture. A summary of the CFU-GM index for these cultures is shown in Table 2.

Colony Forming Unit (CFU) Index

CFU index is calculated by dividing the total number of colonies (CFU-GM, BFU-E and mixed) obtained at day 12 by the total number of colonies obtained at day 0. A CFU index of 1 indicates that the number of colonies at day 12 is equivalent to the number of colonies at the start of the culture. A summary of the CFU index for these cultures is shown in Table 2.

Table 2

Ex-vivo Expansion

Assay	Growth Factor	Donor #1	Donor #2	Donor #3	Donor #4	Donor #5
Proliferation Index of CD34+ Cell Cultures at Day 12	pMON13416	31.2	44.4	13.9	18.3	5
	native hIL-3	9	4.1	3.4	10.7	1.4
Percentage CD15+ Cells at Day 12 of CD34+ Cultures	pMON13416	44	42.4	72.2	48.8	54.5
	native hIL-3	25.6	10.5	43.3	26	18.2
Percentage CD41+ Cells at Day 12 of CD34+ Cultures	pMON13416	9.2	13.7	2.3	1.4	3.1
	native hIL-3	18.9	14.1	13.7	4.2	5.5
Total Number of Megakaryoctes (E+05) In Day 10-12 Cultures	pMON13416	11	24	1.3	1	0.6
	native hIL-3	6.8	2.3	5.9	0.2	0.3
Colony Forming Unit Granulocyte Macrophage (CFU-GM) Index	pMON13416	1.1	2.5	0.06	1	0.1
	native hIL-3	0.2	0.06	0.03	0.03	0.03
Colony Forming Unit-Index	pMON13416	1.6	3.7	0.1	0.8	0.2
	native hIL-3	0.2	0.08	0.03	0.03	0.04

Additional details may be found in co-pending United States Patent Application Serial number 08/411,795 which is hereby incorporated by reference in its entirety as if written herein.

All references, patents or applications cited herein are incorporated by reference in their entirety as if written herein.

Various other examples will be apparent to the person skilled in the art after reading the present disclosure without departing from the spirit and scope of the invention. It is intended that all such other examples be included within the scope of the appended claims.